JOURNAL OF VIROLOGY, Dec. 2003, p. 13042–13052 Vol. 77, No. 24
0022-538X/03/$08.00+0 DOI: 10.1128/JVI.77.24.13042–13052.2003

Macrophage-Tropic Simian/Human Immunodeficiency Virus Chimeras Use CXCR4, Not CCR5, for Infections of Rhesus Macaque Peripheral Blood Mononuclear Cells and Alveolar Macrophages

Tatsuhiko Igarashi,[1] Olivia K. Donau,[1] Hiromi Imamichi,[2] Marie-Jeanne Dumaurier,[3] Reza Sadjadpour,[1] Ronald J. Plishka,[1] Alicia Buckler-White,[1] Charles Buckler,[1] Anthony F. Suffredini,[4] H. Clifford Lane,[5] John P. Moore,[3] and Malcolm A. Martin[1]*

Laboratory of Molecular Microbiology[1] and Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases,[5] and Critical Care Medicine Department, Clinical Center, National Institutes of Health,[4] Bethesda, Maryland 20892; Science Applications International Corp., Frederick, Inc., Frederick, Maryland 21702[2]; and Department of Microbiology and Immunology, Weill Medical College of Cornell University, New York, New York 10021[3]

Received 29 May 2003/Accepted 8 September 2003

After the nearly complete and irreversible depletion of $CD4^+$ T lymphocytes induced by highly pathogenic simian/human immunodeficiency virus chimeric viruses (SHIVs) during infections of rhesus monkeys, tissue macrophages are able to sustain high levels ($>10^6$ viral RNA copies/ml) of plasma viremia for several months. We recently reported that the virus present in the plasma during the late macrophage phase of infection had acquired changes that specifically targeted the V2 region of gp120 (H. Imamichi et al., Proc. Natl. Acad. Sci. USA 99:13813-13818, 2002); some of these SHIV variants were macrophage-tropic (M-tropic). Those findings have been extended by examining the tropic properties, coreceptor usage, and gp120 structure of five independent SHIVs recovered directly from lymph nodes of late-stage animals. All of these tissue-derived SHIV isolates were able to infect alveolar macrophages. These M-tropic SHIVs used CXCR4, not CCR5, for infections of rhesus monkey PBMC and primary alveolar macrophages. Because the starting highly pathogenic T-tropic SHIV inoculum also utilized CXCR4, these results indicate that the acquisition of M-tropism in the SHIV-macaque system is not accompanied by a change in coreceptor usage. Compared to the initial T-tropic SHIV inoculum, tissue-derived M-tropic SHIVs from individual infected animals carry gp120s containing similar changes (specific amino acid deletions, substitutions, and loss of N-linked glycosylation sites), primarily within the V1 and/or V2 regions of gp120.

In vivo, the principal target of human immunodeficiency virus type 1 (HIV-1) is the $CD4^+$ T cell. Over time, virus-induced elimination and/or dysfunction of this T-lymphocyte subset, whether caused directly or indirectly, leads to clinical disease in infected individuals (10). Like all other lentiviruses, HIV-1 is also able to infect macrophage, a property recognized since the beginning of the AIDS epidemic (21, 50). The pathogenic consequences of HIV-1-infected macrophages is best exemplified by the AIDS dementia complex in which viral RNA expressed in microglia within the central nervous system may cause severe neuronal injury leading to encephalopathy (24). In addition, because of their reported resistance to the cytopathic effects elicited by HIV-1 and long life span, tissue macrophages have also been considered to be an important reservoir of virus (36). In this regard, macrophages have been reported to be a prominent source of virus during the late stages of disease, especially in conjunction with opportunistic infections (35).

HIV-1 infection of macrophages in vivo has been logistically difficult to study. For example, it is not currently known whether monocyte precursors are initially infected in the bone marrow and only begin to produce virus after their migration to and differentiation in specific tissues or whether they become infected after they reach their final tissue of residence. Current understanding about the dynamics of virus production by tissue macrophage is similarly limited: are progeny virions generated as a result of de novo infections or by the continuous release of particles by long-lived cells? Consequently, most present knowledge about HIV-1 infection of macrophage accrues from an in vitro surrogate: monocyte-derived macrophage (MDM). MDM have been useful for identifying so-called macrophage-tropic (M-tropic) HIV-1 strains. M-tropic strains infect MDM and primary $CD4^+$ T lymphocytes but not most human T-cell lines in vitro, fail to induce syncytium formation, and can be recovered from infected persons during all phases of their disease (7, 13, 42). Prototypic M-tropic strains of HIV-1 use the CCR5 chemokine receptor for cell entry (1, 11).

From studies of highly pathogenic SHIVs that irreversibly and systemically deplete $CD4^+$ T lymphocytes in rhesus monkeys within weeks of virus inoculation, we previously reported that tissue macrophages are able to sustain high levels of plasma viremia ($>10^6$ RNA copies/ml) in the virtual absence of $CD4^+$ T cells (16). It was subsequently found that viral variants, circulating in the plasma during the macrophage phase of SHIV infections, carried gp120 V2 changes (specific

* Corresponding author. Mailing address: Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bldg. 4, Rm. 315, 4 Center Dr., MSC 0460, National Institutes of Health, Bethesda, MD 20892. Phone: (301) 496-4012. Fax: (301) 402-0226. E-mail: malm@nih.gov.

double amino acid deletions and the loss of a conserved glycosylation site) (19). Some of these SHIV variants had also acquired the capacity to infect alveolar macrophages (AM). In the present study, the gp120 structure, cell tropism, and coreceptor utilization properties of macrophage-phase simian immunodeficiency virus/HIV chimeras (SHIVs), isolated directly from lymphoid tissues, were examined. In contrast to plasma virus, the tissue-associated SHIVs bore envelope glycoproteins that were genetically more heterogeneous and contained specific changes primarily within V1 and V2. Small molecule coreceptor-targeted inhibitors, specific for CCR5 or CXCR4, were used to assess the chemokine receptor usage by (i) the starting highly pathogenic, T-cell-deleting SHIVs and the (ii) late-phase M-tropic SHIV variants. Blockade of CXCR4 potently suppressed infection of rhesus monkey PBMC by both viruses, whereas CCR5 targeted inhibitors had little if any effect. Infection of macaque AM by M-tropic SHIVs was also suppressed by CXCR4 not by CCR5 specific inhibitors. Thus, the acquisition of macrophage tropism by SHIVs, present in late-phase monkeys, is not accompanied by a switch in chemokine coreceptor usage.

MATERIALS AND METHODS

Virus. The propagation and properties of the highly pathogenic $SHIV_{DH12R}$ and its $SHIV_{DH12R\text{-}PS1}$ derivative have been described previously (12, 17, 51). $SHIV_{DH12R\text{-}CL\text{-}7}$, a highly pathogenic molecular clone of $SHIV_{DH12R\text{-}PS1}$, was cloned from Hirt DNA prepared from $SHIV_{DH12R\text{-}PS1}$-infected rhesus macaque peripheral blood mononuclear cells (PBMC) by using a lambda phage vector (19). Stocks of SIV_{mac239} and SIV_{mac316} used in tissue culture experiments were prepared from supernatants of HeLa cells transfected with infectious molecular clones of each virus (29, 31).

Animal experiments. Rhesus macaques were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (*Guide for the Care and Use of Laboratory Animals* [NIH 85-23], Department of Health and Human Services, 1985) and were housed in a biosafety level 2 facility; biosafety level 3 practices were followed. Phlebotomies and virus inoculations were performed with animals anesthetized with Tiletamine-HCl and Zolazepam-HCl (Telazol; Fort Dodge Laboratories, Fort Dodge, Iowa). Lymphocyte subset analyses and plasma viral load determinations were performed as previously described (12).

Virus isolation from animals chronically infected with $SHIV_{DH12R}$. Specific lymph nodes were collected aseptically from each animal at the time of necropsy (891631 [colonic], AP47 [mesenteric], AG18 [axillary], BD83 [inguinal], and WBJ [mesenteric and colonic]). The lymph nodes were initially minced with scissors, disaggregated by using a Medimachine (BD Biosciences, San Diego, Calif.), and then filtered through a 70-μm-pore-size cell strainer (Falcon 2350; Becton Dickinson, Franklin Lakes, N.J.). After a wash with phosphate-buffered saline, the cells were resuspended in RPMI 1640 medium (Cambrex Bio Science, Walkersville, Md.), supplemented with 20% fetal bovine serum (HyClone, Logan, Utah), 10% human serum type AB (Sigma, St. Louis, Mo.), 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μg of gentamicin/ml at a concentration of 10^7 cells/ml, dispensed into 24-well culture plates, and cultivated in a CO_2 incubator (37°C, 5% CO_2). Nonadherent cells were removed daily by gently replacing the entire culture medium on days 1 to 6. After the removal of the culture medium on day 7, the remaining adherent cells were cocultivated with the same number of uninfected rhesus PBMC. The cocultures were maintained for an additional 8 days, and the entire culture medium was replaced on days 10 to 14. Virus production was assessed by ^{32}P-labeled reverse transcriptase (RT) assay of the culture supernatants (52), and the sample with the highest activity was used as a stock virus, after its titration in MT-4 cells (43).

Virus replication assay in monkey PBMC and AM. The preparation and infection of rhesus monkey PBMC have been previously described (19). Rhesus AM were prepared from uninfected donor animals by bronchoalveolar lavage by using a pediatric bronchoscope (Olympus BF3C40; Olympus America, Inc., Melville, N.Y.). The lavage fluid was filtered through a 70-μm-pore-size cell strainer and centrifuged, and the cell pellet washed four times with 1% bovine serum albumin–phosphate-buffered saline. The cells (10^6 cells/ml) were resuspended in Dulbecco modified Eagle medium (500 μl, total) supplemented with 10% human serum type AB, 5% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μg of gentamicin/ml and dispensed into four-well chamber slides (Labtek II chamber slide system; Nalge Nunc International, Naperville, Ill.) at 5×10^5 cells/well. On days 1 and 2, nonadherent cells were removed by gentle pipetting. After the removal of medium on day 2, the cultured AM were infected with 8×10^3 50% tissue culture infective dose(s) ($TCID_{50}$) of each virus stock and maintained for up to 25 days postinfection with total replacement of the medium every other day. Virus replication was assessed by RT assay of the culture supernatant as described above.

Analysis of SHIV *env* genes. Extraction of viral RNA from individual SHIV stocks and nested RT-PCR amplification of a 3-kbp fragment encompassing the entire *env* gene has been previously reported (17–19). V2 length polymorphism assays were conducted on fluorescently labeled PCR products that had been separated on an ABI 377 DNA sequencer (PE Applied Biosystems) and sized as previously described (19, 20). For V1 length analyses, two outer primers, V12-51(+) (5′-GATGCATGAGGATATAATCAGTTTATGGG-3′) and V12-52(−) (5′-CCTAATTCCATGTGTACATTGTACTGT-3′), were used (57). Two inner primers, V12-50(+) (5′-CCATGTGTAAAATTAACCCCACTCTGTGT-3′) (57) and V1R(−) (5′-GAAAGAGCAGTTTTTTAT-3′), were used for the second-round amplification.

Effect of coreceptor-specific inhibitors on SIV and SHIV replication. TAK-779 (2) and AMD3100 (9) were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. AD101 (47) was provided by Bahige Baroudy (Schering-Plough Research Institute, Bloomfield, N.J.). Chemokine coreceptor usage in PBMC was determined as described previously (58) with minor modifications. Briefly, uninfected rhesus PBMC were prepared as described above. On day 3, 5×10^4 cells were dispensed in 96-well round-bottom plates. Various concentrations (0, 0.05, 0.1, 0.5, 1, 5, and 10 μM) of small-molecule CCR5- or CXCR4-specific inhibitors (AMD3100, TAK-779, and AD101) were added to duplicate wells and incubated for 1 h at 37°C. After this incubation, each test virus was spinoculated (34) at 1,200 × *g* for 1 h at a multiplicity of infection (MOI) of 0.1. On day 5 postinfection, virus replication was assessed by RT assay of the culture supernatants.

Freshly prepared rhesus monkey AM were dispensed into 96-well flat-bottom plates (4×10^4 cells/well) to measure coreceptor usage by SHIVs during macrophage infections. On day 2 postplating, the same concentrations of the AD101 or AMD3100 inhibitor described above to block infections of PBMC were added to quadruplicate wells, followed by incubation for 1 h at 37°C. After this preincubation, 640 $TCID_{50}$ of test virus were added to each well (MOI = 0.016). As a negative control, AM were also inoculated with the T-cell-tropic SIV_{mac239}, in the absence of coreceptor inhibitors. Since $SHIV_{DH12R}$-derived viruses replicate in AM with somewhat faster kinetics than SIV_{mac316} (19), the effects of coreceptor-targeted inhibitors on SHIV and SIV_{mac316} infections were assessed on days 10 and 12, respectively. Virus replication was monitored by determining the RT activity released into the culture fluid. No appreciable replication of SIV_{mac239} in AM was detected, as reported previously (29).

RESULTS

Recovery of M-tropic SHIVs from lymph node specimens. Five rhesus monkeys, inoculated with the original $SHIV_{DH12R}$ swarm (17) or its $SHIV_{DH12R\text{-}PS1}$ (51) and $SHIV_{DH12R\text{-}CL\text{-}7}$ (19) derivatives, all experienced a rapid, irreversible, and systemic depletion of $CD4^+$ T lymphocytes and high levels of plasma viremia (Fig. 1). Animals AG18, BD83, and WBJ were euthanized 17 to 28 weeks postinfection because of marked weight loss, intractable diarrhea, and/or evidence of opportunistic infections; macaques 891631 and AP47 were sacrificed prior to the development of clinical symptoms at weeks 7 and 9. At the time of necropsy, specific lymph nodes were collected from each animal (891631 [colonic], AP47 [mesenteric], AG18 [axillary], BD83 [inguinal], and WBJ [mesenteric and colonic]). Suspensions of each were plated in 24-well culture plates. Nonadherent cells were removed over the next 6 days and, on day 7, the remaining adherent cells were cocultivated with PBMC from naive rhesus monkeys. Virus production was first de-

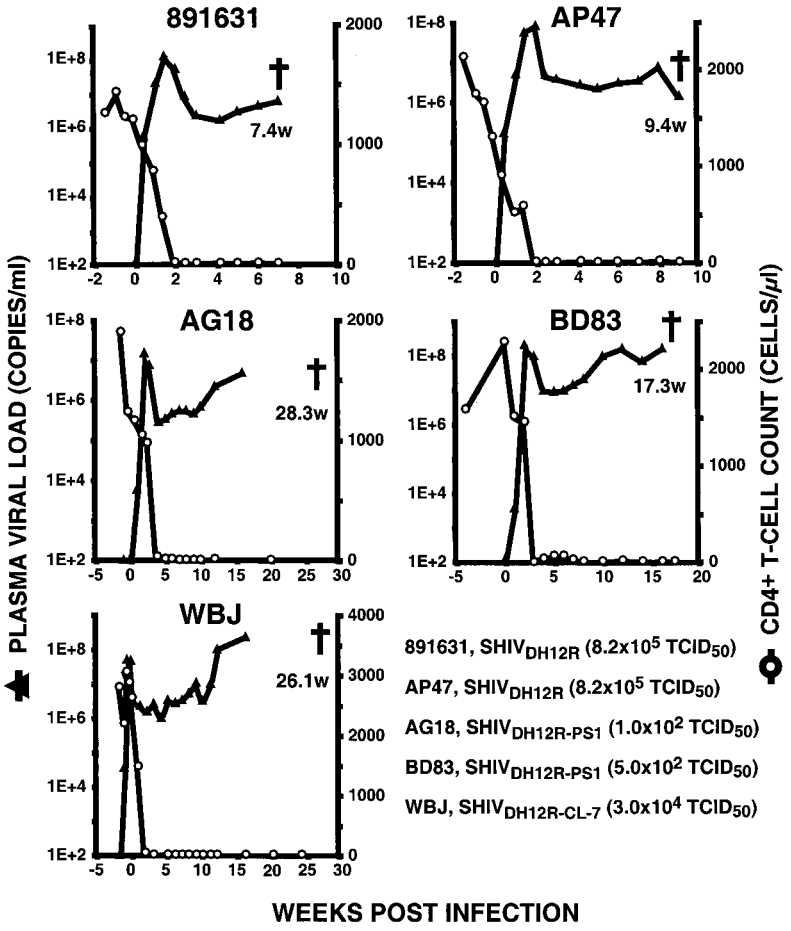

FIG. 1. Viral RNA loads in plasma and peripheral blood CD4$^+$-T-cell profiles of SHIV-infected rhesus monkeys. Each animal was inoculated with the indicated amounts of SHIV$_{DH12R}$, SHIV$_{DH12R-PS1}$, or SHIV$_{DH12R-CL-7}$ intravenously. Viral RNA levels in plasma and the peripheral blood CD4$^+$-T-cell numbers were measured at the indicated times. Daggers indicate the time of euthanasia.

tected on day 3 postcocultivation by measuring the RT activity released into the medium. Culture supernatants were collected daily, and the titers of samples containing the highest RT activity were determined in MT-4 cells and used as virus stocks.

The infectivities of SHIVs recovered from the different adherent lymph node cultures were initially assessed by spinoculation of virus into rhesus PBMC at an MOI of 0.01. As shown in Fig. 2A, three of the four virus controls (SIV$_{mac239}$, SHIV$_{DH12R}$, and SHIV$_{DH12-CL-7}$) achieved peak virus production on day 4 postinfection; SIV$_{mac316}$ was delayed by 1 day. Extensive syncytium formation was observed in the SHIV$_{DH12R}$ and SHIV$_{DH12-CL-7}$-infected PBMC cultures, whereas SIV$_{mac316}$ generated only a few syncytia and SIV$_{mac239}$ induced none. The infection kinetics of the SHIVs, isolated from adherent lymph node cells, in PBMC are shown in Fig. 2B. Compared to the input SHIV$_{DH12R}$ and SHIV$_{DH12-CL-7}$ used for monkey inoculations, all of the tissue-derived SHIVs replicated to high levels but were delayed by 1 or 2 days in reaching peak virus production. Prominent syncytium formation was observed in all of the infected PBMC cultures except those exposed to the WBJ isolate.

Because of logistic problems attending the preparation of sufficient numbers of MDM from PBMC collected from 5- to 6-kg rhesus monkeys, M tropism was assessed by measuring the infectivity of macaque AM collected by bronchoalveolar lavage. Among the control viruses, only SIV$_{mac316}$, previously reported to be M-tropic (29), successfully infected AM (Fig. 3A); no progeny virion production was detected in cultures infected with SIV$_{mac239}$ or the two highly pathogenic SHIVs, SHIV$_{DH12R}$, and SHIV$_{DH12-CL-7}$. In contrast, all five of the SHIVs, isolated directly from lymph nodes of late-stage rhesus monkeys, readily infected AM, and each exhibited faster replication kinetics than SIV$_{mac316}$ (Fig. 3B). Multinucleated giant cells were observed in the SIV$_{mac316}$, and all of the SHIV-infected AM cultures except WBJ (not shown). Taken together, these results indicate that during CD4$^+$-T-cell-depleting infections of rhesus monkeys, the SHIVs recovered from lymph nodes had acquired the capacity to infect tissue macrophage.

Tissue-derived M-tropic SHIVs contain altered gp120 V1, V2, and V3 loops. We previously reported that accompanying the transition from the T cell to the macrophage phase of infection, the SHIVs present in plasma carried envelope glycoproteins containing specific amino acid substitutions and double amino acid deletions within the V2 loop (19). No changes were observed in other regions of gp120. The V2

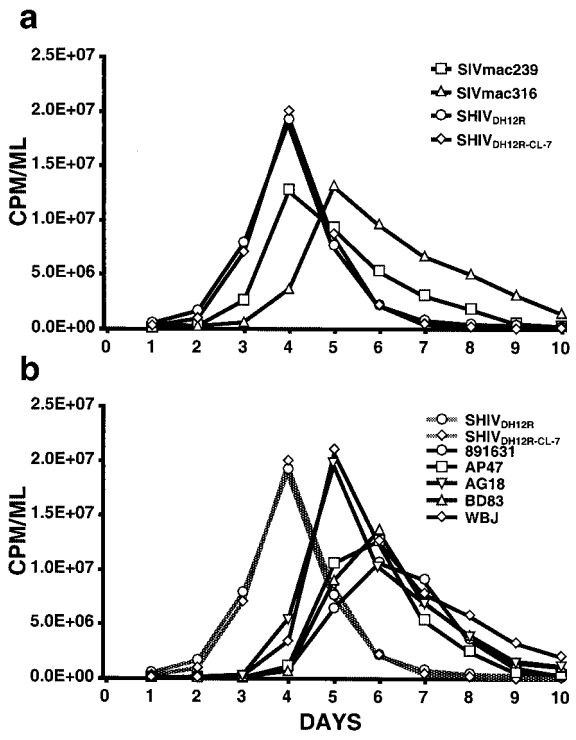

FIG. 2. SHIV and SIV replication in rhesus monkey PBMC. The replication kinetics of virus controls (a) or SHIVs recovered from lymph nodes of animals during the macrophage phase of SHIV infections (b) are shown. Culture supernatants were collected at the indicated time points, and the RT activity was determined. The data shown in the two panels were obtained from the same experiment.

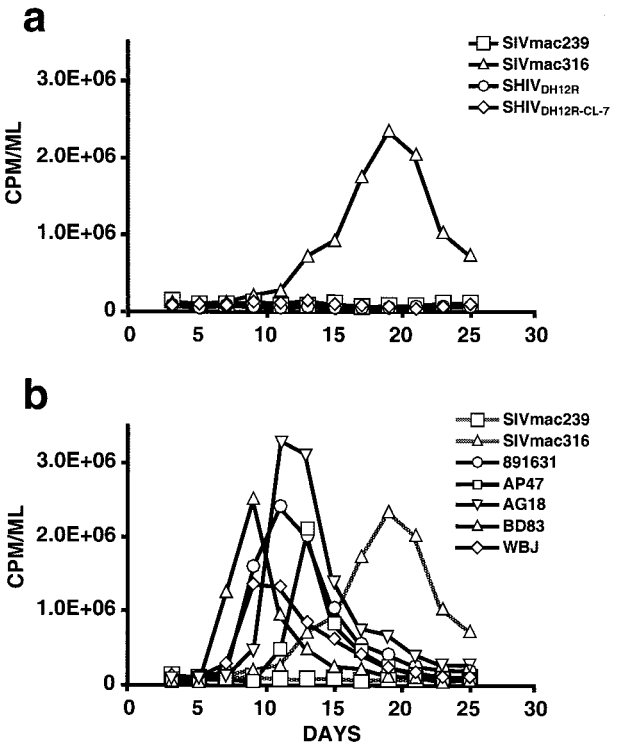

FIG. 3. SHIV and SIV replication in primary monkey AM. The replication kinetics of virus controls (a) or SHIVs recovered from lymph nodes of animals during the macrophage phase of SHIV infections (b) are shown. Culture supernatants were collected at the indicated time points, and the RT activity was measured. The data shown the two panels were obtained from the same experiment.

regions of these plasma-derived viruses had sustained deletions affecting residues 164 to 165 or residues 186 to 187; some of these gp120 molecules had also lost the highly conserved V2 glycosylation site at position 197 (see the "Late Phase Plasma" sequences in Fig. 4). A subset of the late-phase SHIV variants replicated to high levels in cultured AM (19).

To assess whether the M-tropic virus recovered directly from adherent lymph node cells carried similarly altered gp120 envelope glycoproteins, 3-kbp segments encompassing the entire *env* gene were PCR amplified from each of the five SHIV stocks, and the nucleotide sequences of six to eight independent PCR clones were determined. Like the plasma virus present during the macrophage phase, the envelope glycoproteins of the lymph node-derived viruses from monkeys 891631 and AP47 also carried double amino acid deletions affecting residues 164 to 165 and residues 186 to 187 of their respective gp120 V2 loops (Fig. 4, top). However, in contrast to the plasma virus, which had acquired changes only affecting V2, several of the tissue-derived viruses also contained a large six-amino acid-deletion (residues 138 to 143) and loss of the glycosylation site at residue 141 in their V1 regions. In the two monkeys (AG18 and BD83) inoculated with the PS1 derivative of $SHIV_{DH12R}$ (Fig. 4, middle), the changes detected primarily affected V2 and involved deletions (one to five residues) of amino acids mapping to positions 162 to 166 or the loss of the glycosylation site at position 197. A single gp120 PCR clone from animal AG18 also carried the six-amino-acid deletion, previously found in the V1 loop of the two $SHIV_{DH12R}$-infected macaques. WBJ was the only animal inoculated with the molecularly cloned derivative of $SHIV_{DH12R}$, $SHIV_{DH12R\text{-}CL\text{-}7}$. Interestingly, the tissue-derived virus recovered from this monkey contained no gp120 V2 changes (Fig. 4, bottom). Instead, three of eight gp120s bore the same previously observed six-amino-acid deletion in V1, and the remaining five had each lost one of the two V1 N-linked glycosylation sites (at residues 135 or 141).

To independently confirm the V1 and V2 changes in the gp120s of uncloned virus recovered directly from lymph nodes, a fluorescence-amplified fragment length polymorphism assay capable of sampling the entire SHIV population and detecting minor gp120 variants present at low frequencies was used. As shown in Fig. 5, four of the five late-phase SHIVs contained a diverse array of gp120s, with deletions of one to five amino acids affecting their respective V2 loops compared to the starting $SHIV_{DH12R}$ inoculum. This length polymorphism analysis of V2 regions was in general agreement with the sequencing data shown in Fig. 4. A similar analysis of the SHIVs isolated from monkey WBJ was also consistent with the sequencing results: no changes were detected in V2, whereas nearly 50% of the gp120 molecules carried the six-amino-acid deletion in V1. The same V1 deletion was present as a minor gp120 population in the other four SHIVs, although it did represent more than a quarter of the molecules in virus recovered from monkey AP47.

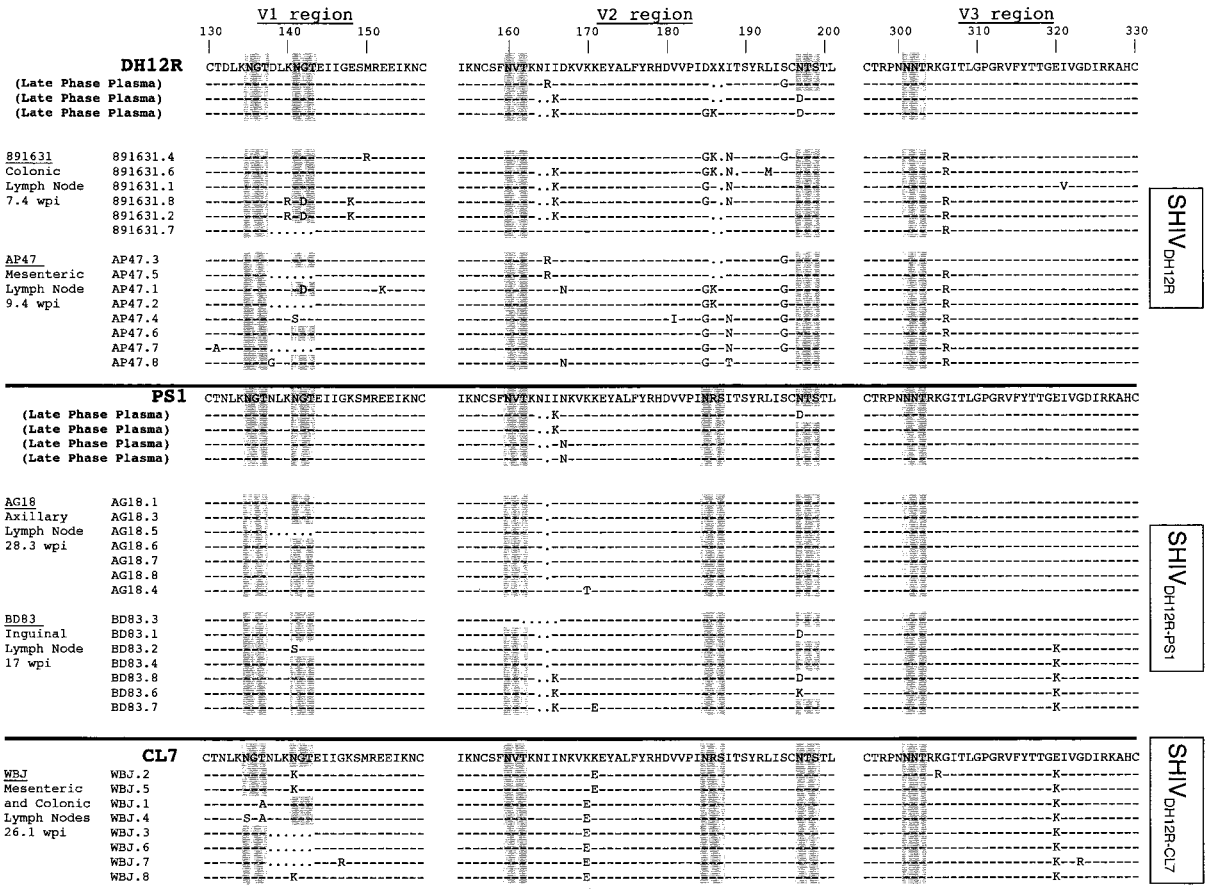

FIG. 4. gp120 V1, V2, and V3 sequence alignments of M-tropic SHIVs recovered from lymph nodes of macrophage-phase animals. *env* gene segments of 3 kbp were RT-PCR amplified from the five indicated lymph node-derived virus stocks. Six to eight independent PCR clones were sequenced from animals originally inoculated with $SHIV_{DH12R}$ (top), $SHIV_{DH12R\text{-}PS1}$ (middle), and $SHIV_{DH12R\text{-}CL7}$ (bottom). The previously described (19) gp120 V1, V2 and V3 sequences, associated with SHIVs present in the plasma of late-stage monkeys infected with $SHIV_{DH12R}$ or $SHIV_{DH12R\text{-}PS1}$, are also shown. Identical residues are indicated by a dash, deleted residues are indicated by a period, and potential N-linked glycosylation sites are shaded.

The alignments presented in Fig. 4 also show that four of the five lymph node-derived M-tropic SHIVs carry gp120s containing a basic amino acid substitution in V3 that is not present in their T-tropic parental virus. Depending on the SHIV inoculum, these changes occurred at either of two locations within V3: G306R or E320K.

T-tropic and M-tropic SHIVs both utilize CXCR4 for entry into rhesus monkey PBMC and AM. The acquisition of M tropism, coupled with the emergence of changes affecting the gp120 V1/V2 structure, raised the possibility that coreceptor utilization by $SHIV_{DH12R}$ and its derivatives had also been altered. This eventuality was examined by using small molecule coreceptor-targeted inhibitors, specific for CCR5 or CXCR4, to assess the coreceptor dependence of replication in rhesus macaque PBMC. In these experiments, virus was spinoculated onto PBMC in the presence of an inhibitor, and progeny virus production was measured as the RT activity released into the medium on day 5 postinfection. Infections of monkey PBMC by SIV_{mac239} and SIV_{mac316} were both blocked by two different CCR5 inhibitors (TAK-779 and AD-101) but not by the CXCR4 inhibitor AMD3100 (Fig. 6a and b). For SIV_{mac239}, this result is in agreement with a previous report showing that CCR5 is the predominant coreceptor used by this virus in macaque PBMC (58). When the assay was performed with three different DH12-related SHIVs ($SHIV_{DH12}$, $SHIV_{DH12R}$, and $SHIV_{DH12R\text{-}CL\text{-}7}$), the opposite outcome was observed: infection of PBMC was completely blocked by AMD 3100 and not by the CCR5 inhibitors (Fig. 6c to e). Some competition was observed with TAK-779, but only at the highest concentrations tested (5 to 10 μM), which might be due to nonspecific effects of this compound. Nonetheless, the 50% inhibitor concentration for TAK-779 against SIV_{mac239} and SIV_{mac316} was, at a minimum, at least 2 orders of magnitude lower than that needed to inhibit the $SHIV_{DH12R}$ variants. Together, these results indicate that the two SIV reference viruses utilize CCR5 and the three T-tropic SHIVs use CXCR4 to enter and spread through cultured rhesus PBMC. The same coreceptor-targeted inhibitors were used to evaluate the infection of PBMC by M-tropic SHIVs. As can be seen in Fig. 7, all were effectively blocked by AMD3100 even at the lowest concentration (0.05 μM) tested. In contrast, neither of the CCR5 inhibitors blocked these infections except, in a few instances, at the highest concentration (10 μM) tested, which again might represent nonspecific effects.

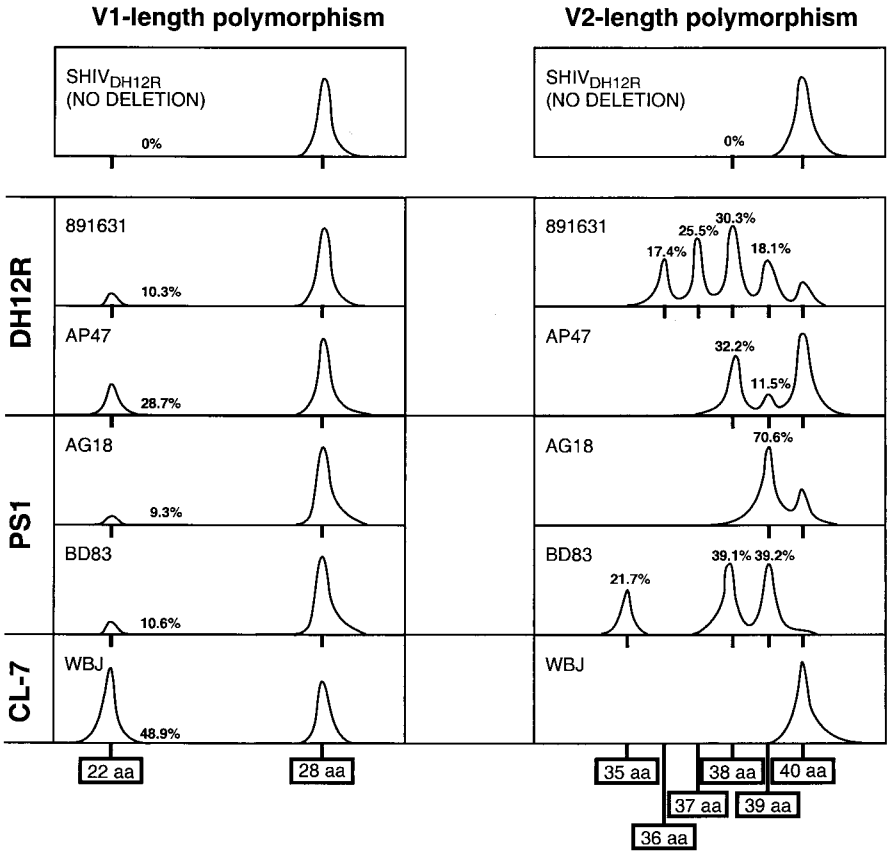

FIG. 5. V1 and V2 loop length polymorphism analyses of SHIVs recovered from lymph nodes during the macrophage phase of infection. Fluorescently labeled V1 or V2 PCR products, amplified from the five indicated SHIV stocks, were separated on a DNA sequencing gel, and their sizes were determined as described in Materials and Methods. The molecularly cloned $SHIV_{DH12R\text{-}CL\text{-}7}$ inoculum, which contains full-length V1 (28 residues) and V2 (40 residues) regions, was used as a reference (top). The percentage of each deleted species in the virus stock was calculated from the fluorescence intensity of each isoform divided by the total intensity.

As noted above, we have defined M-tropism for both SIV and SHIV as the capacity to infect AM rather than MDM. Since the critical question in these analyses of M-tropic SHIVs was to ascertain the coreceptor used for entry into macrophages, not PBMC, an inhibition assay using AM was performed. Because of the logistic difficulties associated with collecting, preparing, and culturing sufficient numbers of AM for replicate assays, two representative M-tropic SHIVs were selected for this analysis. One, the SHIV recovered from animal 891631, which was originally infected with the uncloned $SHIV_{DH12R}$, contained gp120s with V2 loops of variable length (Fig. 4 and 5). The second was the late-stage SHIV isolated from monkey WBJ, initially inoculated with the molecularly cloned $SHIV_{DH12R\text{-}CL\text{-}7}$, and carried a gp120 with a six-amino-acid deletion in the V1 loop and a V2 region of normal size. The entry of SIV_{mac316} into AM was also evaluated in the same system. As shown in Fig. 8, the replication of SIV_{mac316} was completely inhibited by the CCR5-specific inhibitor, AD101. In contrast, the replication of the two M-tropic SHIVs in AM was blocked by the CXCR4 inhibitor AMD3100, whereas the CCR5 inhibitor AD101 had a slight augmenting effect on both viruses. Thus, despite acquiring tropism for AM and acquiring gp120 changes affecting the V1, V2, and V3 regions, all five of the SHIVs recovered from adherent lymph node cells from late-stage animals continued to use CXCR4 for infections of rhesus PBMC. In assays involving AM, the two SHIVs tested also used CXCR4 and not CCR5 to generate progeny virions. Taken together, these experiments show that a coreceptor switch did not accompany a change in SHIV tropism.

DISCUSSION

Three important findings have emerged from these experiments. (i) M-tropic SHIVs utilize CXCR4, not CCR5, to enter rhesus monkey PBMC and AM. (ii) Acquisition of the M-tropic phenotype by highly pathogenic T-tropic SHIVs is not accompanied by a change in chemokine coreceptor usage. (iii) Alterations in variable loops (primarily within V1 and/or V2) are associated with the conversion of T to M tropism, defined in the present study as the capacity of SHIVs to productively infect PBMC or AM.

In studies of cultured MDM, HIV-1 M tropism is usually associated with usage of the CCR5 chemokine receptor (1, 11). However, some primary HIV-1 isolates (viz. dualtropic X4 strains) have been reported to use CXCR4 to enter MDM (44, 49, 55, 56). Similarly, an HIV-1 isolate from a CCR5Δ32 homozygote has recently been shown to use CXCR4 during infections of MDM obtained from both CCR5Δ32/Δ32 and

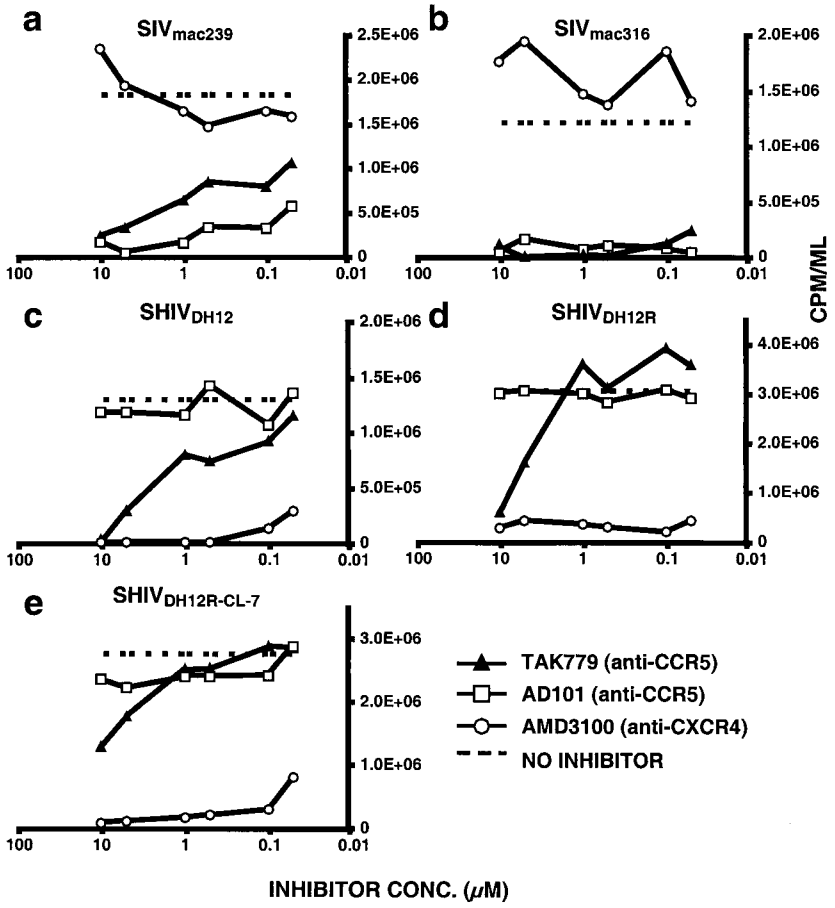

FIG. 6. Coreceptor inhibitor sensitivity of the three SHIV inocula and SIV controls. The inoculum viruses, $SHIV_{DH12R}$, $SHIV_{DH12R\text{-}PS1}$, or $SHIV_{DH12R\text{-}CL\text{-}7}$ were spinoculated onto rhesus PBMC in the presence of the indicated small molecule coreceptor inhibitors. The inhibitor concentrations used were 0.05, 0.1, 0.5, 1.0, 5.0, and 10 μM. The RT activity released into the medium on day 5 postinfection was determined in the absence (dashed line) or presence of inhibitor. SIV_{mac239} and SIV_{mac316} were also analyzed as representative of R5 viruses that can infect macaque PBMC.

CCR5 wild-type donors (32). In addition, a recent survey of HIV-1 strains recovered from brain and lymphoid tissue found that two highly M-tropic isolates entered microglia via CXCR4 and concluded that M tropism rather than coreceptor usage, is predictive of HIV-1 neurotropism (14). Although the results reported in the present study clearly show that SHIVs, isolated directly from adherent lymph node cells of late-stage monkeys, utilize CXCR4 to enter cultured rhesus monkey PBMC and AM, the mechanism used by primate lentiviruses to enter cells of macrophage lineage in vivo is currently unknown. Although it is likely that endocytosis triggered by receptor engagement also mediates virus entry in vivo, it is possible that infections of macrophages are mediated by macropinocytosis or after phagocytosis of virus-infected T lymphocytes (25, 26), neither of which require binding to receptors at the cell surface. Whether this early step in the virus life cycle targets undifferentiated mononuclear cells in the bone marrow or blood or targets differentiated macrophages located in perivascular areas or tissue parenchyma is also unknown.

Our previous analysis of the virus present in plasma during the macrophage phase of pathogenic SHIV infections revealed the existence of envelope glycoproteins with unique amino acid substitutions and deletions limited to the V2 region of gp120 (19). In the present study of SHIVs isolated directly from adherent lymph node cells of late-stage animals, we identified a greater diversity of gp120s, most of which contained specific changes affecting the V1 and V3 regions, as well as V2. Compared to contemporary plasma virus, the SHIVs in lymphoid tissue carried gp120s that were genetically more heterogeneous. For example, mixed SHIV populations, bearing V2 regions with up to five amino acid deletions, were present in the lymph nodes of individual monkeys (Fig. 5), whereas virus in plasma carried gp120s with only double amino acid deletions in V2 (19). The large six-amino-acid V1 deletion, never previously observed in the envelope glycoproteins of circulating late-stage SHIVs, was present in three of the five tissue-derived viruses and comprised nearly 50% of the SHIV recovered from animal WBJ (Fig. 5).

In discussing the emergence of M-tropic SHIVs containing specific gp120 changes, we previously noted the presence of V2 variants in the starting virus inocula and suggested that the M-tropic viruses might have arisen as a result of the selected outgrowth of preexisting virions in the uncloned $SHIV_{DH12R}$ and $SHIV_{DH12R\text{-}PS1}$ stocks used (19). In the present study,

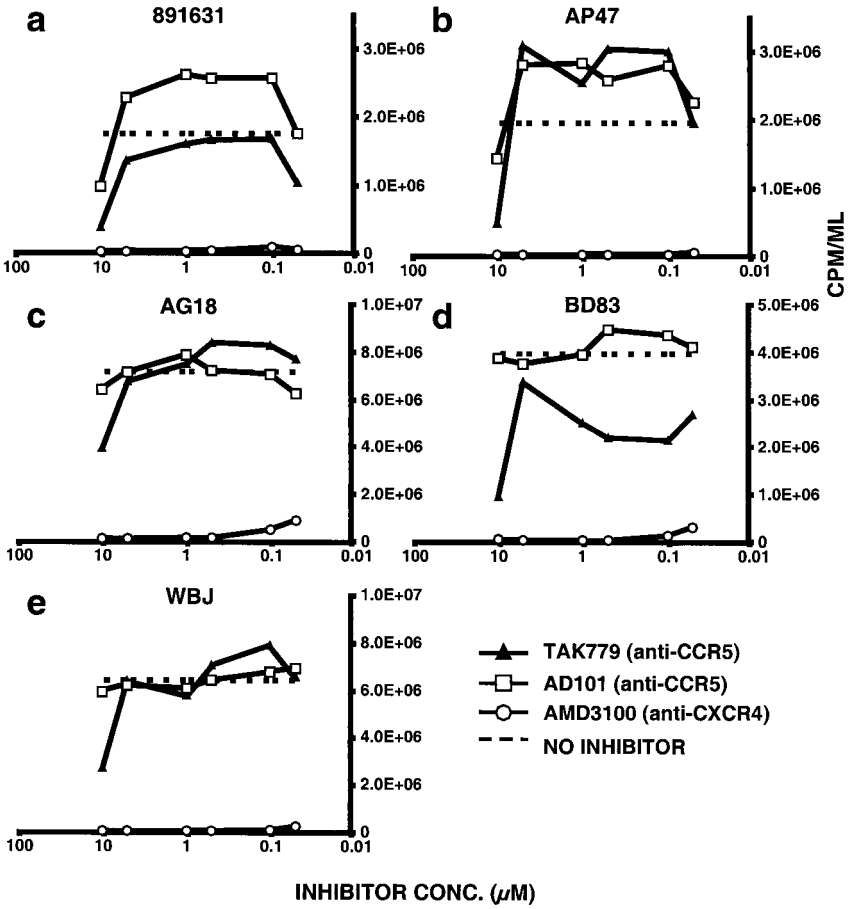

FIG. 7. Coreceptor usage of five M-tropic SHIVs for entry into macaque PBMC. The indicated SHIVs, all isolated from lymph nodes of late-stage animals, were spinoculated onto rhesus PBMC in the presence of the indicated small molecule coreceptor inhibitors. Progeny virus production was monitored as described in the legend to Fig. 6.

monkey WBJ was inoculated with the molecularly cloned $SHIV_{DH12R\text{-}CL\text{-}7}$; M-tropic variants could not have preexisted in this virus inoculum and would have only appeared during the course of its evolution in vivo, as the selected product of error-prone reverse transcription. Intriguingly, the major gp120 changes in late stage M-tropic virions isolated from the lymphoid tissue of macaque WBJ were located in V1 and not in V2 (Fig. 4). In the eight independent *env* PCR clones from this animal, the V1 region contained either a six-amino-acid deletion or the loss of one of the two glycosylation sites. The V2 change, which did appear, was the conversion of the basic amino acid lysine at positions 170 or 171 to glutamic acid in every *env* gene amplified (Fig. 4).

The functional implications of gp120s with partial deletions or the loss of an N-linked glycosylation site affecting V1 or V2 are currently unknown. SHIVs bearing specifically altered V1 and V2 regions are being constructed, and their receptor binding and infectivity properties will be assessed. Nonetheless, it is now appreciated that during HIV-1 entry, the binding of virions to CD4 induces a conformational change in V1/V2 and a repositioning of the V1/V2 stem (23, 37, 40, 45, 54). It is quite possible that the deletions and/or the elimination of a bulky glycan from either the V1 or the V2 regions of M-tropic SHIVs significantly alters the quaternary structure of the envelope trimeric complex and unmasks the CD4 and/or coreceptor binding sites on gp120. In the context of acquiring M tropism, it is now recognized that levels of CD4, CCR5, and CXCR4 expression on the surface of human AM and CD4 levels on rhesus monkey AM are extremely low to undetectable (30, 53). Thus, the capacity to infect macaque AM may be limited to SIVs and SHIVs carrying unique gp120s, which are able to mediate fusion with cells expressing very little surface CD4 and/or chemokine receptor. In this regard, the restriction of MDM to infection by the T-lymphocyte-tropic SIV_{mac239} strain has been shown to be due to low levels of cell surface CD4; this block could be inhibited by overexpressing human or simian CD4 (4).

The entry of M-tropic SHIVs into cells, which express extremely low levels of CD4, may also be facilitated by the introduction of a basic amino acid within the V3 loop, a change (G306R or E320K) observed in four of the five gp120s analyzed (Fig. 4). HIV-1 particles have been reported to attach to heparin sulfate proteoglycans via positively charged residues on V3 prior to their binding to CD4 (5, 28, 38). This low-affinity interaction, which is perhaps augmented by the V3 changes observed in the gp120s of M-tropic SHIVs, could

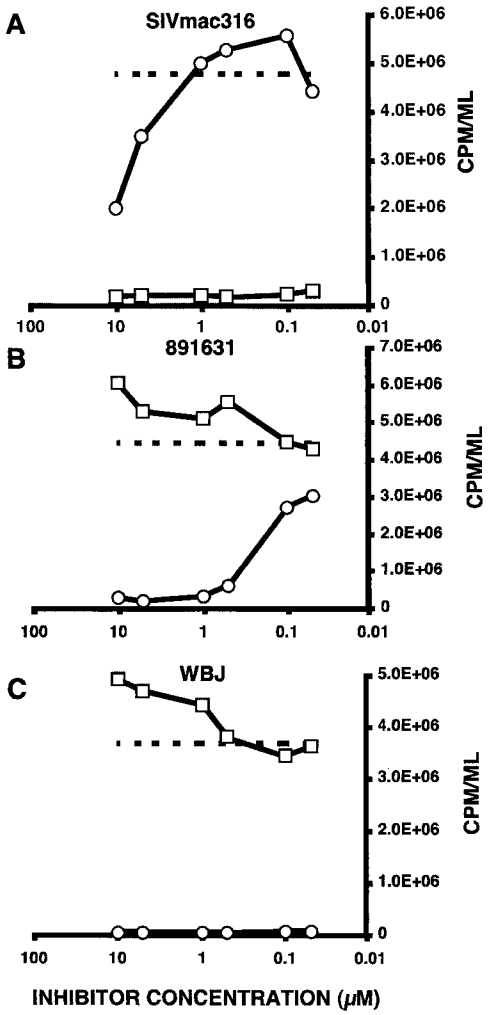

FIG. 8. M-tropic SHIVs use CXCR4 for infections of rhesus monkey AM. SHIVs, recovered from lymph nodes of animals 891631 or WBJ and SIV_{mac316} were used to infect macaque AM in the presence of AD101 or AMD3100 coreceptor inhibitors. Progeny virus production was monitored on day 10 for M-tropic SHIVs and on day 12 for SIV_{mac316} by the RT activity released into the culture fluid. Symbols: □, AD101 (CCR5 inhibitor); ○, AMD3100 (CXCR4 inhibitor); - -, no inhibitor.

facilitate two-dimensional surface scanning for the presence of the negligible amounts of CD4 on AM.

The results obtained with inhibitors that target specific chemokine receptors raise another important issue pertaining to the unusually rapid, $CD4^+$-T-cell-depleting disease associated with the highly pathogenic SHIVs compared to the much-slower-paced immunodeficiencies induced by SIV and HIV-1. The results indicate, quite conclusively, that the T-tropic $SHIV_{DH12R}$ and its derivatives exclusively use CXCR4 for infections of rhesus monkey PBMC, whereas T-tropic SIV strains, such as SIV_{mac239}, exhibit exactly the opposite property: they use CCR5 to enter monkey PBMC (Fig. 6). In the case of HIV-1, M-tropic CCR5-utilizing strains are commonly detected in recently infected individuals, although CXCR4 variants can be recovered from many patients during the later symptomatic phase of the infection (8, 39, 41, 59). When one considers that the fraction of circulating $CD4^+$ T lymphocytes expressing CXCR4 is very high (>80%) and the fraction expressing CCR5 is quite low (5 to 10%) in both human and macaque PBMC (15, 48; Y. Nishimura, unpublished results), the complete and systemic elimination of $CD4^+$ T lymphocytes induced by SHIVs could simply be attributed to the targeting and unrelenting depletion of the very abundant CXCR4-expressing $CD4^+$ T cells. Although it could be argued that the extremely aggressive phenotype exhibited by the pathogenic SHIVs is an aberrant characteristic of this macaque model of AIDS, some of its features have also been reported for HIV-1 infections of humans. For example, CXCR4-utilizing strains have been recovered from rare CCR5Δ32/Δ32 homozygotes shortly after the establishment of an HIV-1 infection (3, 6, 33). These individuals frequently experience a rapidly progressive clinical course characterized by a marked loss of $CD4^+$ T lymphocytes similar to that seen during acute infections of rhesus monkeys with highly pathogenic SHIVs (22, 27, 46). The low $CD4^+$-T-cell levels in these patients fail to rise after the institution of highly active antiretroviral therapy. Taken together, one might conclude that SHIV-induced immunodeficiency of macaques is a CXCR4 disease, whereas HIV-1 and SIV primarily cause AIDS by infecting CCR5-bearing cells. The former is associated with a rapid clinical course and the complete, systemic, and irreversible loss of $CD4^+$ T cells, while the latter causes a considerably slower but unrelenting disease with symptoms of immunodeficiency that do not require the total elimination of this lymphocyte subset.

ACKNOWLEDGMENTS

We thank Frances Banks, Sekou Savane, Wes Thornton, and Russ Byrum for devoted animal care; Randy Elkins for his help in procuring and maintaining animals, Dusty Rhodes for invaluable assistance in performing bronchoalveolar lavage; Bahige Baroudy and Jayaram Tagat, Schering-Plough Research Institute, for providing AD101; and Robert E. Means and Ronald C. Desrosiers for helpful suggestions about culturing AM.

REFERENCES

1. **Alkhatib, G., C. Combadiere, C. C. Broder, Y. Feng, P. E. Kennedy, P. M. Murphy, and E. A. Berger.** 1996. CC CKR5: a RANTES, MIP-1α, MIP-1β receptor as a fusion cofactor for macrophage-tropic HIV-1. Science **272:** 1955–1958.
2. **Baba, M., O. Nishimura, N. Kanzaki, M. Okamoto, H. Sawada, Y. Iizawa, M. Shiraishi, Y. Aramaki, K. Okonogi, Y. Ogawa, K. Meguro, and M. Fujino.** 1999. A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity. Proc. Natl. Acad. Sci. USA **96:**5698–5703.
3. **Balotta, C., P. Bagnarelli, M. Violin, A. L. Ridolfo, D. Zhou, A. Berlusconi, S. Corvasce, M. Corbellino, M. Clementi, M. Clerici, M. Moroni, and M. Galli.** 1997. Homozygous Δ32 deletion of the CCR5 chemokine receptor gene in an HIV-1-infected patient. AIDS **11:**F67–F71.
4. **Bannert, N., D. Schenten, S. Craig, and J. Sodroski.** 2000. The level of CD4 expression limits infection of primary rhesus monkey macrophages by a T-tropic simian immunodeficiency virus and macrophagetropic human immunodeficiency viruses. J. Virol. **74:**10984–10993.
5. **Batinic, D., and F. A. Robey.** 1992. The V3 region of the envelope glycoprotein of human immunodeficiency virus type 1 binds sulfated polysaccharides and CD4-derived synthetic peptides. J. Biol. Chem. **267:**6664–6671.
6. **Biti, R., R. Ffrench, J. Young, B. Bennetts, G. Stewart, and T. Liang.** 1997. HIV-1 infection in an individual homozygous for the CCR5 deletion allele. Nat. Med. **3:**252–253.
7. **Collman, R., N. F. Hassan, R. Walker, B. Godfrey, J. Cutilli, J. C. Hastings, H. Friedman, S. D. Douglas, and N. Nathanson.** 1989. Infection of monocyte-derived macrophages with human immunodeficiency virus type 1 (HIV-1): monocyte-tropic and lymphocyte-tropic strains of HIV-1 show distinctive patterns of replication in a panel of cell types. J. Exp. Med. **170:**1149–1163.
8. **Connor, R. I., K. E. Sheridan, D. Ceradini, S. Choe, and N. R. Landau.** 1997. Change in coreceptor use correlates with disease progression in HIV-1-infected individuals. J. Exp. Med. **185:**621–628.

9. **Donzella, G. A., D. Schols, S. W. Lin, J. A. Este, K. A. Nagashima, P. J. Maddon, G. P. Allaway, T. P. Sakmar, G. Henson, E. De Clercq, and J. P. Moore.** 1998. AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. Nat. Med. **4:**72–77.
10. **Douek, D. C., L. J. Picker, and R. A. Koup.** 2003. T cell dynamics in HIV-1 infection. Annu. Rev. Immunol. **21:**265–304.
11. **Dragic, T., V. Litwin, G. P. Allaway, S. R. Martin, Y. Huang, K. A. Nagashima, C. Cayanan, P. J. Maddon, R. A. Koup, J. P. Moore, and W. A. Paxton.** 1996. HIV-1 entry into $CD4^+$ cells is mediated by the chemokine receptor CC-CKR-5. Nature **381:**667–673.
12. **Endo, Y., T. Igarashi, Y. Nishimura, C. Buckler, A. Buckler-White, R. Plishka, D. S. Dimitrov, and M. A. Martin.** 2000. Short- and long-term clinical outcomes in rhesus monkeys inoculated with a highly pathogenic chimeric simian/human immunodeficiency virus. J. Virol. **74:**6935–6945.
13. **Gartner, S., and M. Popovic.** 1990. Macrophage tropism of HIV-1. AIDS Res. Hum. Retrovir. **6:**1017–1021.
14. **Gorry, P. R., G. Bristol, J. A. Zack, K. Ritola, R. Swanstrom, C. J. Birch, J. E. Bell, N. Bannert, K. Crawford, H. Wang, D. Schols, E. de Clercq, K. Kunstman, S. M. Wolinsky, and D. Gabuzda.** 2001. Macrophage tropism of human immunodeficiency virus type 1 isolates from brain and lymphoid tissues predicts neurotropism independent of coreceptor specificity. J. Virol. **75:**10073–10089.
15. **Grivel, J.-C., and L. B. Margolis.** 1999. CCR5- and CXCR4-tropic HIV-1 are equally cytopathic for their T-cell targets in human lymphoid tissue. Nat. Med. **5:**344–346.
16. **Igarashi, T., C. R. Brown, Y. Endo, A. Buckler-White, R. Plishka, N. Bischofberger, V. Hirsch, and M. A. Martin.** 2001. Macrophage are the principal reservoir and sustain high virus loads in rhesus macaques after the depletion of $CD4^+$ T cells by a highly pathogenic simian immunodeficiency virus/HIV type 1 chimera (SHIV): implications for HIV-1 infections of humans. Proc. Natl. Acad. Sci. USA **98:**658–663.
17. **Igarashi, T., Y. Endo, G. Englund, R. Sadjadpour, T. Matano, C. Buckler, A. Buckler-White, R. Plishka, T. Theodore, R. Shibata, and M. Martin.** 1999. Emergence of a highly pathogenic simian/human immunodeficiency virus in a rhesus macaque treated with anti-CD8 mAb during a primary infection with a nonpathogenic virus. Proc. Natl. Acad. Sci. USA **96:**14049–14054.
18. **Imamichi, H., K. A. Crandall, V. Natarajan, M. K. Jiang, R. L. Dewar, S. Berg, A. Gaddam, M. Bosche, J. A. Metcalf, R. T. Davey, Jr., and H. C. Lane.** 2001. Human immunodeficiency virus type 1 quasi species that rebound after discontinuation of highly active antiretroviral therapy are similar to the viral quasi species present before initiation of therapy. J. Infect. Dis. **183:**36–50.
19. **Imamichi, H., T. Igarashi, T. Imamichi, O. K. Donau, Y. Endo, Y. Nishimura, R. L. Willey, A. F. Suffredini, H. C. Lane, and M. A. Martin.** 2002. Amino acid deletions are introduced into the V2 region of gp120 during independent pathogenic simian immunodeficiency virus/HIV chimeric virus (SHIV) infections of rhesus monkeys generating variants that are macrophage tropic. Proc. Natl. Acad. Sci. USA **99:**13813–13818.
20. **Jansson, M., E. Backstrom, G. Scarlatti, A. Bjorndal, S. Matsuda, P. Rossi, J. Albert, and H. Wigzell.** 2001. Length variation of glycoprotein 120 V2 region in relation to biological phenotypes and coreceptor usage of primary HIV type 1 isolates. AIDS Res. Hum. Retrovir. **17:**1405–1414.
21. **Koenig, S., H. E. Gendelman, J. M. Orenstein, M. C. Dal Canto, G. H. Pezeshkpour, M. Yungbluth, F. Janotta, A. Aksamit, M. A. Martin, and A. S. Fauci.** 1986. Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy. Science **233:**1089–1093.
22. **Kuipers, H., C. Workman, W. Dyer, A. Geczy, J. Sullivan, and R. Oelrichs.** 1999. An HIV-1-infected individual homozygous for the CCR-5 delta32 allele and the SDF-1 3′A allele. AIDS **13:**433–434.
23. **Kwong, P. D., R. Wyatt, J. Robinson, R. W. Sweet, J. Sodroski, and W. A. Hendrickson.** 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature **393:**648–659.
24. **Lipton, S. A., and H. E. Gendelman.** 1995. Seminars in medicine of the Beth Israel Hospital, Boston: dementia associated with the acquired immunodeficiency syndrome. N. Engl. J. Med. **332:**934–940.
25. **Liu, N. Q., A. S. Lossinsky, W. Popik, X. Li, C. Gujuluva, B. Kriederman, J. Roberts, T. Pushkarsky, M. Bukrinsky, M. Witte, M. Weinand, and M. Fiala.** 2002. Human immunodeficiency virus type 1 enters brain microvascular endothelia by macropinocytosis dependent on lipid rafts and the mitogen-activated protein kinase signaling pathway. J. Virol. **76:**6689–6700.
26. **Marechal, V., M.-C. Prevost, C. Petit, E. Perret, J.-M. Heard, and O. Schwartz.** 2001. Human immunodeficiency virus type 1 entry into macrophages mediated by macropinocytosis. J. Virol. **75:**11166–11177.
27. **Michael, N. L., J. A. Nelson, V. N. KewalRamani, G. Chang, S. J. O'Brien, J. R. Mascola, B. Volsky, M. Louder, G. C. White II, D. R. Littman, R. Swanstrom, and T. R. O'Brien.** 1998. Exclusive and persistent use of the entry coreceptor CXCR4 by human immunodeficiency virus type 1 from a subject homozygous for CCR5 Δ32. J. Virol. **72:**6040–6047.
28. **Mondor, I., S. Ugolini, and Q. J. Sattentau.** 1998. Human immunodeficiency virus type 1 attachment to HeLa CD4 cells is CD4 independent and gp120 dependent and requires cell surface heparans. J. Virol. **72:**3623–3634.
29. **Mori, K., D. J. Ringler, T. Kodama, and R. C. Desrosiers.** 1992. Complex determinants of macrophage tropism in *env* of simian immunodeficiency virus. J. Virol. **66:**2067–2075.
30. **Mori, K., M. Rosenzweig, and R. C. Desrosiers.** 2000. Mechanisms for adaptation of simian immunodeficiency virus to replication in alveolar macrophages. J. Virol. **74:**10852–10859.
31. **Naidu, Y. M., H. W. Kestler III, Y. Li, C. V. Butler, D. P. Silva, D. K. Schmidt, C. D. Troup, P. K. Sehgal, P. Sonigo, M. D. Daniel, and R. C. Desrosiers.** 1988. Characterization of infectious molecular clones of simian immunodeficiency virus (SIV_{mac}) and human immunodeficiency virus type 2: persistent infection of rhesus monkeys with molecularly cloned SIV_{mac}. J. Virol. **62:**4691–4696.
32. **Naif, H. M., A. L. Cunningham, M. Alali, S. Li, N. Nasr, M. M. Buhler, D. Schols, E. de Clercq, and G. Stewart.** 2002. A human immunodeficiency virus type 1 isolate from an infected person homozygous for CCR5Δ32 exhibits dual tropism by infecting macrophages and MT2 cells via CXCR4. J. Virol. **76:**3114–3124.
33. **O'Brien, T. R., C. Winkler, M. Dean, J. A. E. Nelson, M. Carrington, N. L. Michael, and G. C. White II.** 1997. HIV-1 infection in a man homozygous for CCR5Δ32. Lancet **349:**1219.
34. **O'Doherty, U., W. J. Swiggard, and M. H. Malim.** 2000. Human immunodeficiency virus type 1 spinoculation enhances infection through virus binding. J. Virol. **74:**10074–10080.
35. **Orenstein, J. M., C. Fox, and S. M. Wahl.** 1997. Macrophages as a source of HIV during opportunistic infections. Science **276:**1857–1861.
36. **Pierson, T., J. McArthur, and R. F. Siliciano.** 2000. Reservoirs for HIV-1: mechanisms for viral persistence in the presence of antiviral immune responses and antiretroviral therapy. Annu. Rev. Immunol. **18:**665–708.
37. **Rizzuto, C. D., R. Wyatt, N. Hernandez-Ramos, Y. Sun, P. D. Kwong, W. A. Hendrickson, and J. Sodroski.** 1998. A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. Science **280:**1949–1953.
38. **Roderiquez, G., T. Oravecz, M. Yanagishita, D. C. Bou-Habib, H. Mostowski, and M. A. Norcross.** 1995. Mediation of human immunodeficiency virus type 1 binding by interaction of cell surface heparan sulfate proteoglycans with the V3 region of envelope gp120-gp41. J. Virol. **69:**2233–2239.
39. **Roos, M. T., J. M. Lange, R. E. de Goede, R. A. Coutinho, P. T. Schellekens, F. Miedema, and M. Tersmette.** 1992. Viral phenotype and immune response in primary human immunodeficiency virus type 1 infection. J. Infect. Dis. **165:**427–432.
40. **Sattentau, Q. J., J. P. Moore, F. Vignaux, F. Traincard, and P. Poignard.** 1993. Conformational changes induced in the envelope glycoproteins of the human and simian immunodeficiency viruses by soluble receptor binding. J. Virol. **67:**7383–7393.
41. **Schuitemaker, H., M. Koot, N. A. Kootstra, M. W. Dercksen, R. E. de Goede, R. P. van Steenwijk, J. M. Lange, J. K. Schattenkerk, F. Miedema, and M. Tersmette.** 1992. Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus population. J. Virol. **66:**1354–1360.
42. **Schuitemaker, H., N. A. Kootstra, R. E. de Goede, F. de Wolf, F. Miedema, and M. Tersmette.** 1991. Monocytotropic human immunodeficiency virus type 1 (HIV-1) variants detectable in all stages of HIV-1 infection lack T-cell line tropism and syncytium-inducing ability in primary T-cell culture. J. Virol. **65:**356–363.
43. **Shibata, R., F. Maldarelli, C. Siemon, T. Matano, M. Parta, G. Miller, T. Fredrickson, and M. A. Martin.** 1997. Infection and pathogenicity of chimeric simian-human immunodeficiency viruses in macaques: determinants of high virus loads and CD4 cell killing. J. Infect. Dis. **176:**362–373.
44. **Simmons, G., J. D. Reeves, A. McKnight, N. Dejucq, S. Hibbitts, C. A. Power, E. Aarons, D. Schols, E. de Clercq, A. E. I. Proudfoot, and P. R. Clapham.** 1998. CXCR4 as a functional coreceptor for human immunodeficiency virus type 1 infection of primary macrophages. J. Virol. **72:**8453–8457.
45. **Sullivan, N., Y. Sun, Q. Sattentau, M. Thali, D. Wu, G. Denisova, J. Gershoni, J. Robinson, J. Moore, and J. Sodroski.** 1998. CD4-induced conformational changes in the human immunodeficiency virus type 1 gp120 glycoprotein: consequences for virus entry and neutralization. J. Virol. **72:**4694–4703.
46. **Theodorou, I., L. Meyer, M. Magierowska, C. Katlama, C. Rouzioux, and the Seroco Study Group.** 1997. HIV-1 infection in an individual homozygous for CCR5Δ32. Lancet **349:**1219–1220.
47. **Trkola, A., S. E. Kuhmann, J. M. Strizki, E. Maxwell, T. Ketas, T. Morgan, P. Pugach, S. Xu, L. Wojcik, J. Tagat, A. Palani, S. Shapiro, J. W. Clader, S. McCombie, G. R. Reyes, B. M. Baroudy, and J. P. Moore.** 2002. HIV-1 escape from a small molecule, CCR5-specific entry inhibitor does not involve CXCR4 use. Proc. Natl. Acad. Sci. USA **99:**395–400.
48. **Veazey, R. S., K. G. Mansfield, I. C. Tham, A. C. Carville, D. E. Shvetz, A. E. Forand, and A. A. Lackner.** 2000. Dynamics of CCR5 expression by $CD4^+$ T cells in lymphoid tissues during simian immunodeficiency virus infection. J. Virol. **74:**11001–11007.
49. **Verani, A., E. Pesenti, S. Polo, E. Tresoldi, G. Scarlatti, P. Lusso, A. G. Siccardi, and D. Vercelli.** 1998. CXCR4 is a functional coreceptor for infec-

tion of human macrophages by CXCR4-dependent primary HIV-1 isolates. J. Immunol. **161:**2084–2088.
50. **Wiley, C. A., R. D. Schrier, J. A. Nelson, P. W. Lampert, and M. B. Oldstone.** 1986. Cellular localization of human immunodeficiency virus infection within the brains of acquired immune deficiency syndrome patients. Proc. Natl. Acad. Sci. USA **83:**7089–7093.
51. **Willey, R. L., R. Byrum, M. Piatak, Y. B. Kim, M. W. Cho, J. L. Rossio, Jr., J. Bess, Jr., T. Igarashi, Y. Endo, L. O. Arthur, J. D. Lifson, and M. A. Martin.** 2003. Control of viremia and prevention of simian-human immunodeficiency virus-induced disease in rhesus macaques immunized with recombinant vaccinia viruses plus inactivated simian immunodeficiency virus and human immunodeficiency virus type 1 particles. J. Virol. **77:**1163–1174.
52. **Willey, R. L., D. H. Smith, L. A. Lasky, T. S. Theodore, P. L. Earl, B. Moss, D. J. Capon, and M. A. Martin.** 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J. Virol. **62:**139–147.
53. **Worgall, S., R. Connor, R. J. Kaner, E. Fenamore, K. Sheridan, R. Singh, and R. G. Crystal.** 1999. Expression and use of human immunodeficiency virus type 1 coreceptors by human alveolar macrophages. J. Virol. **73:**5865–5874.
54. **Wyatt, R., J. Moore, M. Accola, E. Desjardin, J. Robinson, and J. Sodroski.** 1995. Involvement of the V1/V2 variable loop structure in the exposure of human immunodeficiency virus type 1 gp120 epitopes induced by receptor binding. J. Virol. **69:**5723–5733.
55. **Yi, Y., S. N. Isaacs, D. A. Williams, I. Frank, D. Schols, E. de Clercq, D. L. Kolson, and R. G. Collman.** 1999. Role of CXCR4 in cell-cell fusion and infection of monocyte-derived macrophages by primary human immunodeficiency virus type 1 (HIV-1) strains: two distinct mechanisms of HIV-1 dual tropism. J. Virol. **73:**7117–7125.
56. **Yi, Y., S. Rana, J. D. Turner, N. Gaddis, and R. G. Collman.** 1998. CXCR-4 is expressed by primary macrophages and supports CCR5-independent infection by dual-tropic but not T-tropic isolates of human immunodeficiency virus type 1. J. Virol. **72:**772–777.
57. **Zhang, L., C. Chung, B.-S. Hu, T. He, Y. Guo, A. J. Kim, E. Skulsky, X. Jin, A. Hurley, B. Ramratnam, M. Markowitz, and D. D. Ho.** 2000. Genetic characterization of rebounding HIV-1 after cessation of highly active antiretroviral therapy. J. Clin. Investig. **106:**839–845.
58. **Zhang, Y.-J., B. Lou, R. B. Lal, A. Gettie, P. A. Marx, and J. P. Moore.** 2000. Use of inhibitors to evaluate coreceptor usage by simian and simian/human immunodeficiency viruses and human immunodeficiency virus type 2 in primary cells. J. Virol. **74:**6893–6910.
59. **Zhu, T., H. Mo, N. Wang, D. S. Nam, Y. Cao, R. A. Koup, and D. D. Ho.** 1993. Genotypic and phenotypic characterization of HIV-1 patients with primary infection. Science **261:**1179–1181.